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## THE LATERAL DISTRIBUTION OF INTRAMEMBRANE PARTICLES IN THE ERYTHROCYTE MEMBRANE AND RECOMBINANT VESICLES

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### Summary

Triton X-100 (in concentrations which did not cause a significant solubilization of membrane material) caused aggregation of the intramembrane particles of human erythrocyte ghosts.

Ghosts from which the extrinsic proteins had been removed by alkali treatment showed a temperature-induced aggregation of the particles. With virtually no spectrin present, the particles in these stripped ghosts could still be aggregated by manipulations with ionic strength and pH, or by the addition of calcium.

Recombinant vesicles were made from a Triton X-100 extract and a mixture of phospholipids with a composition which resembled that of the inner monolayer of erythrocyte membrane. In these recombinants the same manipulations with ionic strength and pH and the addition of calcium caused a rearrangement of the particles, resulting in the appearance of particle-free areas. In recombinants prepared from a Triton X-100 extract and egg phosphatidylcholine the lateral distribution of the particles was not altered by these manipulations.

It is concluded that in the erythrocyte membrane the intramembrane particles can be aggregated by effects of external agents on lipid components. In this light the role of spectrin in stabilizing the membrane by interactions with lipids in the inner monolayer is discussed.

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### Introduction

In the human erythrocyte membrane the proteins and phospholipids are asymmetrically arranged [1–5]. Interaction between the various polypeptides have been shown to result in supramolecular protein ensembles, one involving

the extrinsic proteins spectrin and actin [6,7], another one consisting of the intrinsic protein band 3 \* and the extrinsic proteins band 4.2, band 6 (glyceraldehyde-3-phosphate dehydrogenase) and aldolase [7]. Labeling studies have implicated the major intrinsic proteins band 3 and glycophorin as constituents of the freeze-fracture intramembrane particles [8,9]. The involvement of band 3 has been confirmed by reconstitution studies [10–12], while the role of glycophorin is still uncertain [8,13,14]. Several lines of evidence point to some kind of interaction between spectrin and intrinsic proteins. Perturbation of spectrin by antibodies was shown to affect the distribution of anionic sites at the outer surface of the membrane [15]. *Ricinus communis* lectin, reacting with carbohydrate receptors at the outer surface, was shown to cause an enhanced cross-linking of spectrin [16]. Various treatments were shown to cause aggregation of the intramembrane particles, but only after partial removal of spectrin [17]. A similar aggregation of intramembrane particles could be induced in a reconstituted system by including a spectrin-containing protein fraction [10]. It was concluded that precipitation of spectrin was the actual cause of the observed particle aggregation [10,18,19]. About the nature of the interaction between spectrin and the intrinsic proteins little is known. A spectrin-binding polypeptide has been identified recently [20,21], but its relation to the major intrinsic proteins is still unclear. Cross-linking studies have suggested a structural linkage between the spectrin-actin network and band 3 [22]. Spectrin-actin was also shown to interact with negatively charged phospholipids [23,24], and perturbation of spectrin by oxidizing agents was correlated with changes in the arrangement of phospholipids in the membrane [25].

The experiments presented here were designed to obtain more information about the mechanism of particle aggregation in the erythrocyte membrane. Since we have previously found that Triton X-100 can aggregate the particles [12], the effect of this detergent was investigated more systematically. To evaluate the role of spectrin, the aggregation of particles was also studied in membranes from which essentially all extrinsic proteins had been removed by an alkali treatment [12,26]. The role of phospholipids in the aggregation of the particles was examined using vesicles which were reconstituted from a crude Triton X-100 extract and various phospholipids from the erythrocyte membrane.

## Materials and Methods

Triton X-100 was obtained from Rohm and Haas, Bio Beads SM-2 from Bio-Rad. All other reagents were of analytical grade. Egg phosphatidylcholine was purified according to Singleton et al. [27]. Ghosts were prepared from fresh or recently outdated human blood according to Dodge et al. [28] and stored at  $-20^{\circ}\text{C}$ . Lipids were extracted from ghosts as described by Reed et al. [29]. The lipids were fractionated on a silica gel column [30], and fractions were further purified by CM-cellulose chromatography [31]. The effect of low concentrations of Triton X-100 on the freeze-fracture morphology of ghosts was studied in 18 mM sodium phosphate, pH 8.0. 1 vol. of ghost suspension (3–

\* The major polypeptides of the human erythrocyte membrane are numbered according to Steck [1].

4 mg protein/ml) was mixed with 20 vols. of cold 18 mM sodium phosphate, pH 8.0. The membranes were sedimented at  $30\,000 \times g$  for 10 min and resuspended in 3 vols. of ice-cold Triton X-100 containing (0.01–0.05%, w/v) 18 mM sodium phosphate, pH 8.0. After 20 min incubation on ice the membranes were sedimented at  $30\,000 \times g$  for 30 min and used for freeze-fracture electron microscopy. Aliquots were taken before and after centrifugation for protein, sialic acid, phospholipid and cholesterol determinations.

Stripped ghosts, essentially free of extrinsic proteins, were obtained by a short exposure to pH 12 [12,26]. 1 vol. of ghost suspension was mixed with 20 vols. of cold 50 mM Tris/acetic acid, pH 8.0, and sedimented at  $30\,000 \times g$  for 10 min. The membranes were resuspended in 5 vols. of ice-cold 0.01 M NaOH, immediately sedimented at  $50\,000 \times g$  for 30 min and washed twice. The first washing was with 7.5 vols. of 50 mM Tris/acetic acid, pH 8.0, followed by 30 min centrifugation at  $50\,000 \times g$ ; the second washing was with 20 vols. of 50 mM Tris/acetic acid, pH 8.0, followed by 30 min centrifugation at  $30\,000 \times g$ . The stripped ghosts were incubated either with 150 mM NaCl (pH 8.0), 10 mM  $\text{CaCl}_2$  (pH 8.0) or at pH 5.5. Each incubation was carried out in 10 vols. of the appropriate 50 mM Tris/acetic acid buffer at room temperature for 30 min, followed by 30 min centrifugation at  $30\,000 \times g$  at  $20^\circ\text{C}$ .

A crude Triton X-100 extract from ghosts was prepared as outlined previously [12], with minor modifications. Band 6-depleted membranes from 1 vol. ghost suspension were extracted with 1 vol. 0.5% Triton X-100 in 50 mM Tris/acetic acid pH 8.0, for 20 min on ice. Band 3-containing vesicles were prepared from this Triton X-100 extract as described before [12,32]. Phospholipids were dried from chloroform and dispersed in fresh, ice-cold Triton X-100 extract, to give a concentration of 4 mg/ml. The mixture was slowly rotated overnight with 0.3 g of wet SM-2 beads/ml at  $4^\circ\text{C}$ . The vesicles were sedimented at  $150\,000 \times g$  for 90 min and fused by repeated freezing-thawing of the pellet [12]. Incubations with 150 mM NaCl, 10 mM  $\text{CaCl}_2$  or at pH 5.5 were carried out for 30 min at room temperature in the appropriate 50 mM Tris/acetic acid buffer, followed by 60 min centrifugation at  $150\,000 \times g$  and  $4^\circ\text{C}$ . Some of the reconstitutions were carried out with a concentrated Triton X-100 extract, which was obtained in the following way. A crude Triton X-100 extract was absorbed to DEAE-Sephadex A25 in 50 mM Tris/acetic acid, pH 8.0, 0.5% Triton X-100. The Triton X-100 concentration was lowered to 0.05%, and the bound protein eluted with 400 mM NaCl in 50 mM Tris/acetic acid, pH 8.0, 0.05% Triton X-100. After removal of the NaCl by dialysis this protein solution was concentrated by ultrafiltration, using an Amicon PM10 filter.

A protein fraction mainly consisting of spectrin and actin was extracted from ghosts according to Bennett and Branton [20]. 1 vol. of ghost suspension was mixed with 20 vols. of cold 0.3 mM sodium phosphate, pH 7.4. The membranes were sedimented at  $30\,000 \times g$  for 10 min, resuspended in 2 vols. 0.3 mM sodium phosphate (pH 7.4) and incubated at  $37^\circ\text{C}$  for 20 min with stirring. Membrane residues were sedimented at  $150\,000 \times g$  for 30 min, and the supernatant was used as spectrin-actin solution (approximately 0.6 mg protein/ml). To examine the effect of Triton X-100 on the solubility of spectrin-actin, 0.9 ml of the spectrin-actin solution was mixed with 0.1 ml Triton X-100 containing (0–5%, w/v) 180 mM sodium phosphate, pH 8.0. The mixture was

incubated on ice for 20 min and centrifuged for 30 min at  $100\,000 \times g$ . Aliquots of the supernatants were taken for protein determination.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Steck and Yu [26], using 4.0% acrylamide gels and bromophenol blue as tracking dye.

Freeze-fracture electron microscopy was performed as outlined previously [33]. Glycerol was added to the samples to prevent freeze damage. Spray-freezing was done as described by Bachmann et al. [34].

Protein was determined according to Lowry et al. [35], with 3% sodium dodecyl sulfate in the alkaline copper reagent to prevent interference by Triton X-100. Lipid extractions were carried out according to Renkonen et al. [36]. Phospholipids were separated by two dimensional thin-layer chromatography, using the procedure of Broekhuysse [37]. Phosphorus was measured according to a modification of the Fiske-Subbarow procedure [38], sialic acid according to Warren [39] and cholesterol according to Rudel and Morris [40].

## Results

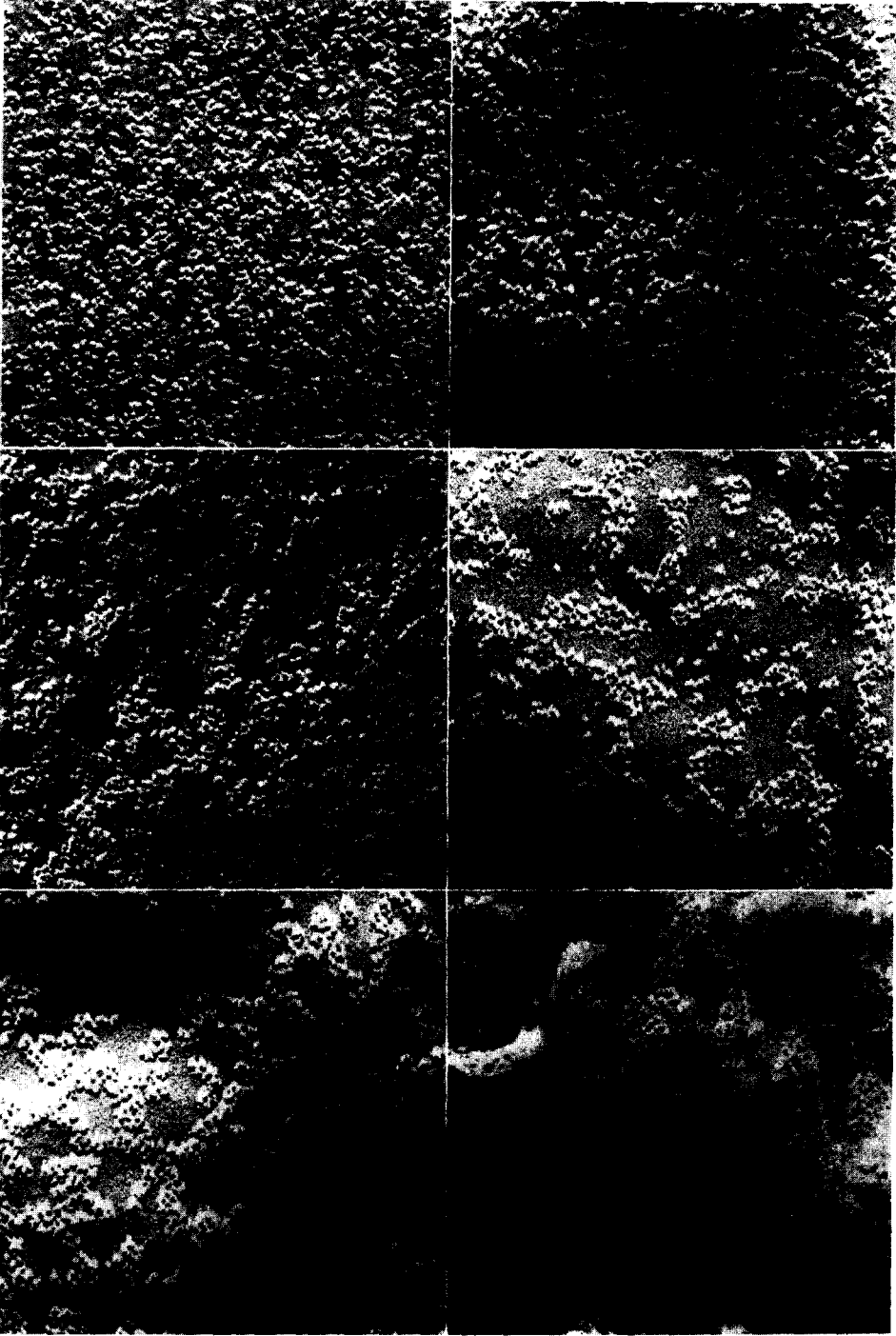
Human erythrocyte membranes were exposed to low concentrations of Triton X-100 (0.01–0.05%, w/v) by incubation on ice in 18 mM sodium phosphate (pH 8.0) for 20 min. As shown in Table I only limited amounts of protein, sialic acid and phospholipid were extracted under these conditions, especially at concentrations below 0.04%. Release of cholesterol could not be detected. Freeze-fracture electron microscopy of the Triton X-100-treated membranes showed a marked aggregation of the intramembrane particles, even after incubation with 0.01% Triton X-100 (Fig. 1A). The extent of aggregation gradually increased with higher detergent concentrations, resulting in a very extensive aggregation pattern after incubation with 0.05% Triton X-100 (Fig. 1). To examine the effect of Triton X-100 on the solubility of spectrin under these conditions, a low ionic strength extract from ghosts, mainly consisting of spectrin and actin (0.6 mg protein/ml) was incubated on ice with Triton X-100 (0–0.5%, w/v) in 18 mM sodium phosphate, pH 8.0. After 30 min centrifugation at  $100\,000 \times g$  65–70% of the protein was recovered in the supernatant. This result did not depend on the detergent concentration,

TABLE I

EXTRACTION OF MEMBRANE MATERIAL FROM ERYTHROCYTE GHOSTS BY LOW CONCENTRATIONS OF TRITON X-100

pH 8.0 and 0°C.

Concn. Triton X-100 (%, w/v)	% extracted		
	Protein	Sialic acid	Phospholipid
0.01	1.3	<3	0.5
0.02	1.9	<3	0.8
0.03	1.9	<3	1.1
0.04	3.3	11	3.1
0.05	4.9	28	4.3



**Fig. 1.** Freeze-fracture electron micrographs of erythrocyte membranes treated with increasing concentrations of Triton X-100 in 18 mM sodium phosphate (pH 8.0) at 0°C. (A) 0%; (B) 0.01%; (C) 0.02%; (D) 0.03%; (E) 0.04%; and (F) 0.05% (w/v). Magnification X100 000.

indicating that spectrin-actin is not precipitated by the detergent under these conditions.

Treatment of ghosts with 0.01 M NaOH removes virtually all extrinsic proteins [12,26]. After 30 min centrifugation at  $50\,000 \times g$  52% of the total protein and about 4% of both phospholipids and cholesterol were recovered in the supernatant. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the residual membrane material showed the presence of only about 2% of the spectrin bands 1 and 2 (Fig. 2). Two dimensional thin-layer chromatography of a lipid extract showed that no lysophospholipids had been formed in detectable quantities. As shown by freeze-fracture electron microscopy, the ghosts fragment into vesicles. These vesicles have mainly an inside-out orientation, as judged from the distribution of the intramembrane particles between the fracture faces [12]. It appeared that the aggregation state of the intramembrane particles in these vesicles is strongly affected by the temperature (Fig. 3). When a sample was quenched from  $23^\circ\text{C}$ , the particles were moderately aggregated (Fig. 3B). The aggregation was more pronounced when the quenching was done from  $0^\circ\text{C}$  (Fig. 3C) and very extensive when quenching was done from  $-20^\circ\text{C}$  (Fig. 3D). Spray-freezing of the vesicles from  $37^\circ\text{C}$ , however, showed a nearly homogeneous distribution of the particles (Fig. 3A).

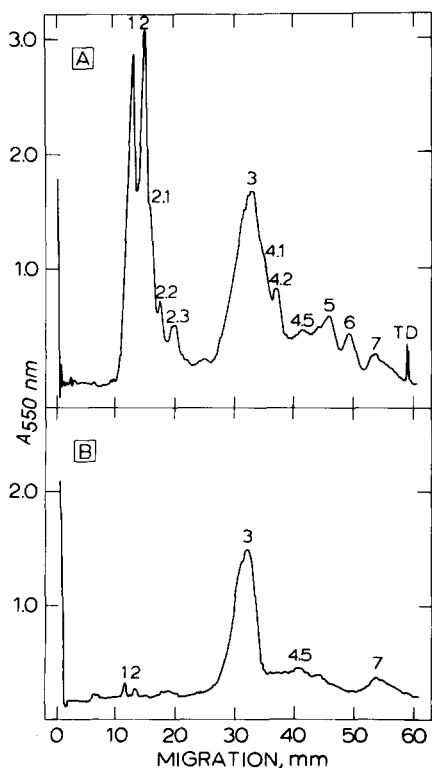


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of erythrocyte membranes (A) and alkali-treated membranes (B). Protein derived from  $20\ \mu\text{l}$  of ghost suspension was applied to 4% polyacrylamide gels. The gels were stained with Coomassie brilliant blue and scanned in a densitometer.

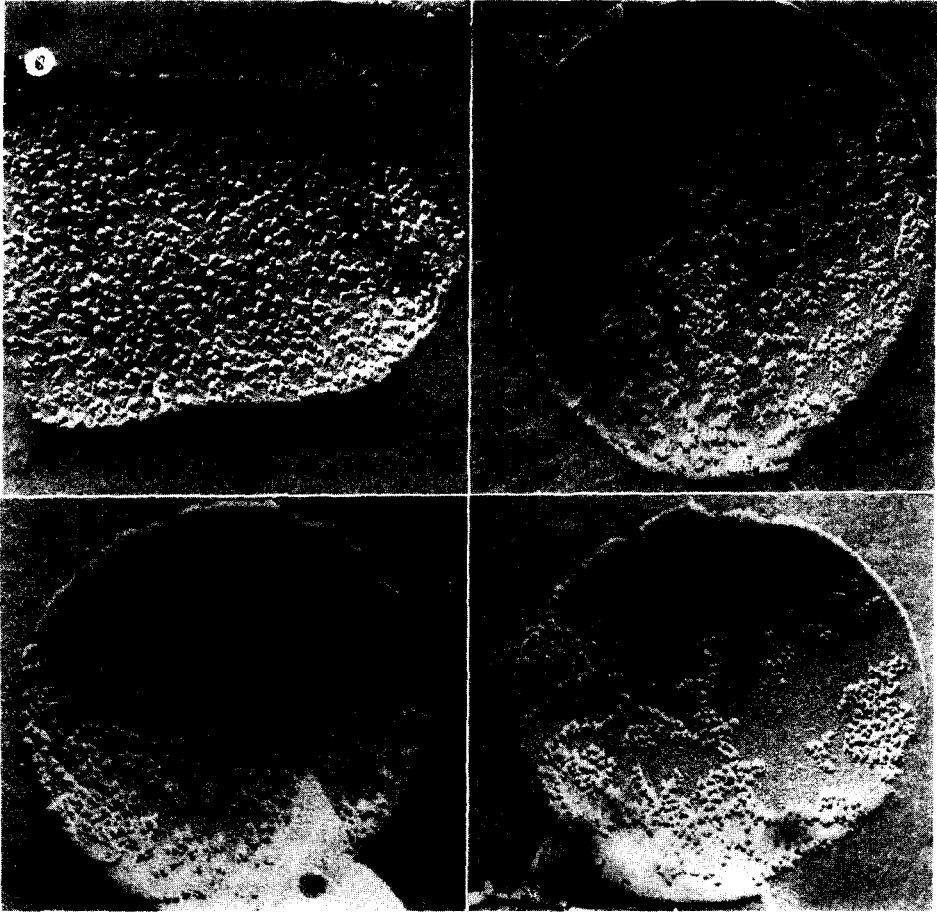


Fig. 3. Freeze-fracture electron micrographs of alkali-treated erythrocyte membranes in 50 mM Tris/acetic acid, pH 8.0, spray-frozen from 37°C (A) and quenched by standard freezing methods from 23°C (B), 0°C (C) and -20°C (D). Magnification X100 000.

Elgsaeter and Branton [17] have shown that after removal of part of the spectrin, the intramembrane particles in ghosts are aggregated by a variety of conditions, including isotonic NaCl, millimolar concentrations of CaCl<sub>2</sub> or low pH. In Fig. 4 it is shown that after stripping off all extrinsic proteins by a 0.01 M NaOH treatment, the particles were still strongly aggregated by incubation in 150 mM NaCl, 10 mM CaCl<sub>2</sub> or a pH 5.5 buffer. This means that the aggregation of the particles under these conditions is not caused by any of the extrinsic proteins.

To investigate whether this kind of particle aggregation could either be caused by an effect on the intrinsic proteins which form the particles, or by an effect on lipid components of the membrane, vesicles showing intramembrane particles were reconstituted from a crude Triton X-100 extract and various phospholipids. A crude Triton X-100 extract was obtained by extracting band 6-depleted ghosts with 0.5% Triton X-100 in 50 mM Tris/acetic acid, pH 8.0, as outlined in Materials and Methods. The extract contained 24% of the total

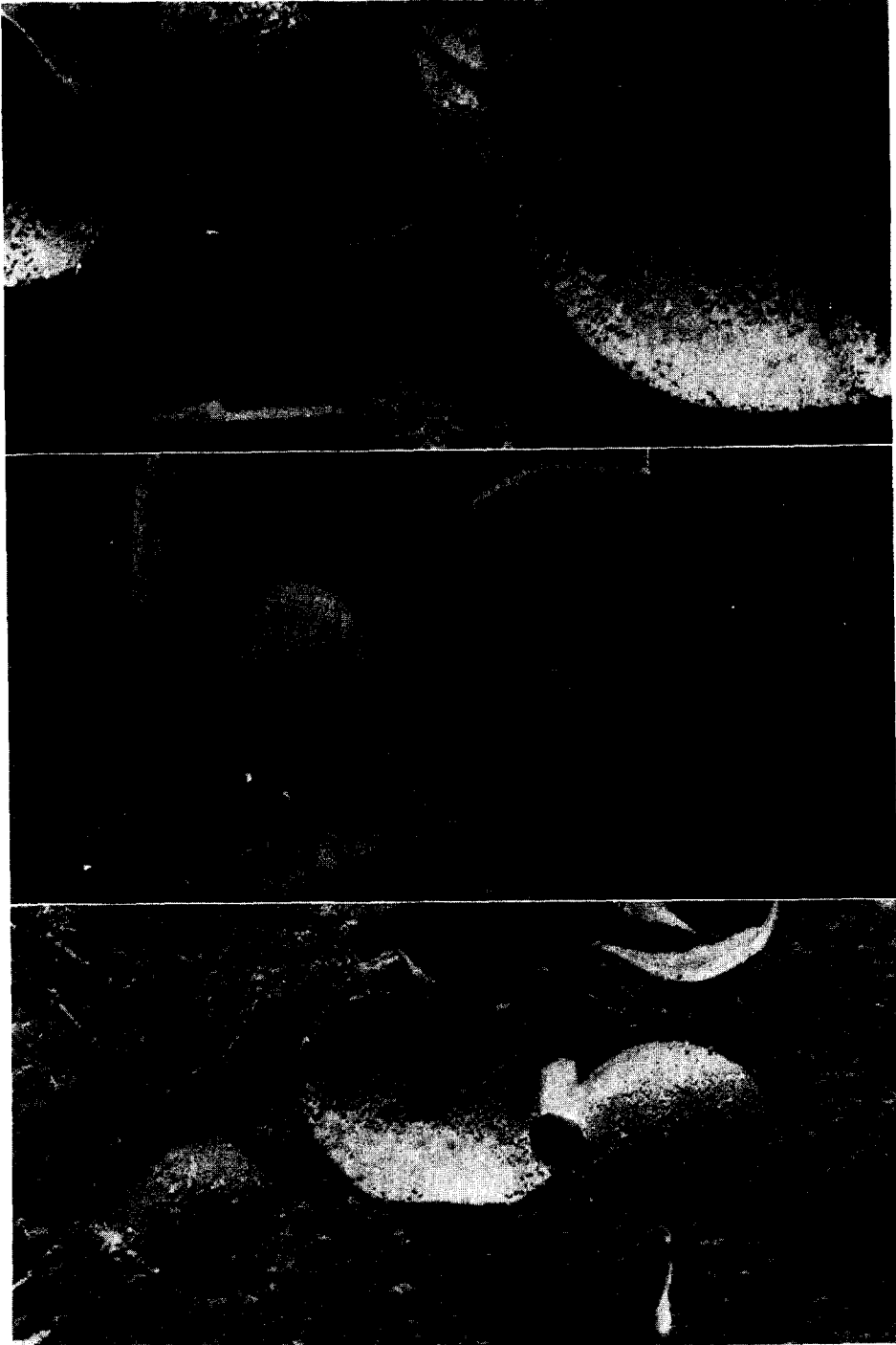


Fig. 4. Freeze-fracture electron micrographs of alkali-treated erythrocyte membranes after incubation for 30 min at room temperature in (A) 150 mM NaCl, 50 mM Tris/acetic acid, pH 8.0; (B) 10 mM  $\text{CaCl}_2$ , 50 mM Tris/acetic acid, pH 8.0, and (C) 50 mM Tris/acetic acid, pH 5.5. Magnification  $\times 100\,000$ .



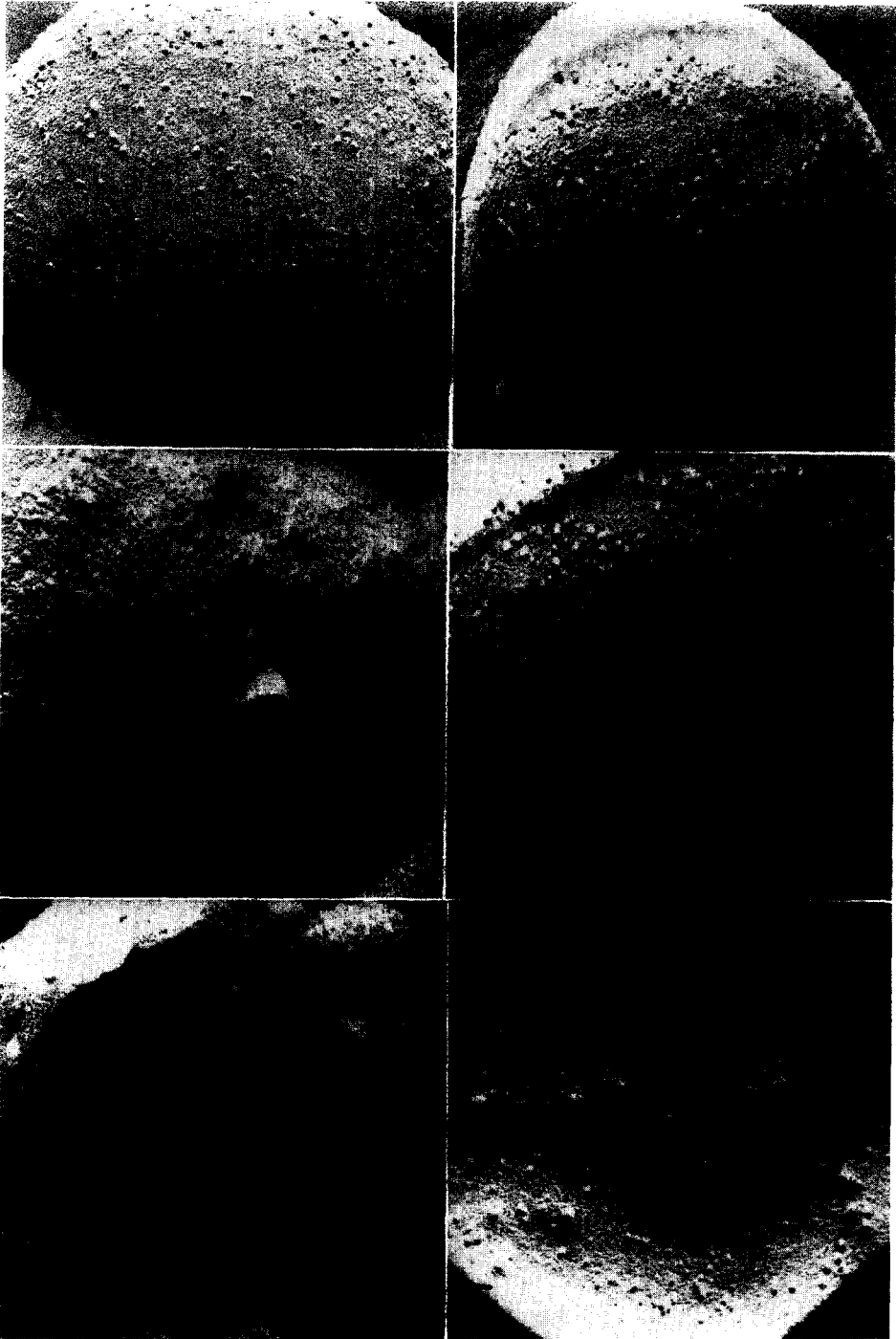


Fig. 5. Freeze-fracture electron micrographs of recombinant vesicles, prepared from a crude Triton X-100 extract and a mixture of phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (2 : 1 : 1) from erythrocyte membranes. The vesicles were fused by repeated freezing and thawing and incubated for 30 min at room temperature in (A) 50 mM Tris/acetic acid, pH 8.0; (B) 150 mM NaCl, 50 mM Tris/acetic acid, pH 8.0; (C) 50 mM Tris/acetic acid, pH 5.5, and (D-F) 10 mM  $\text{CaCl}_2$ , 50 mM Tris/acetic acid, pH 8.0. Magnification  $\times 100\ 000$ .

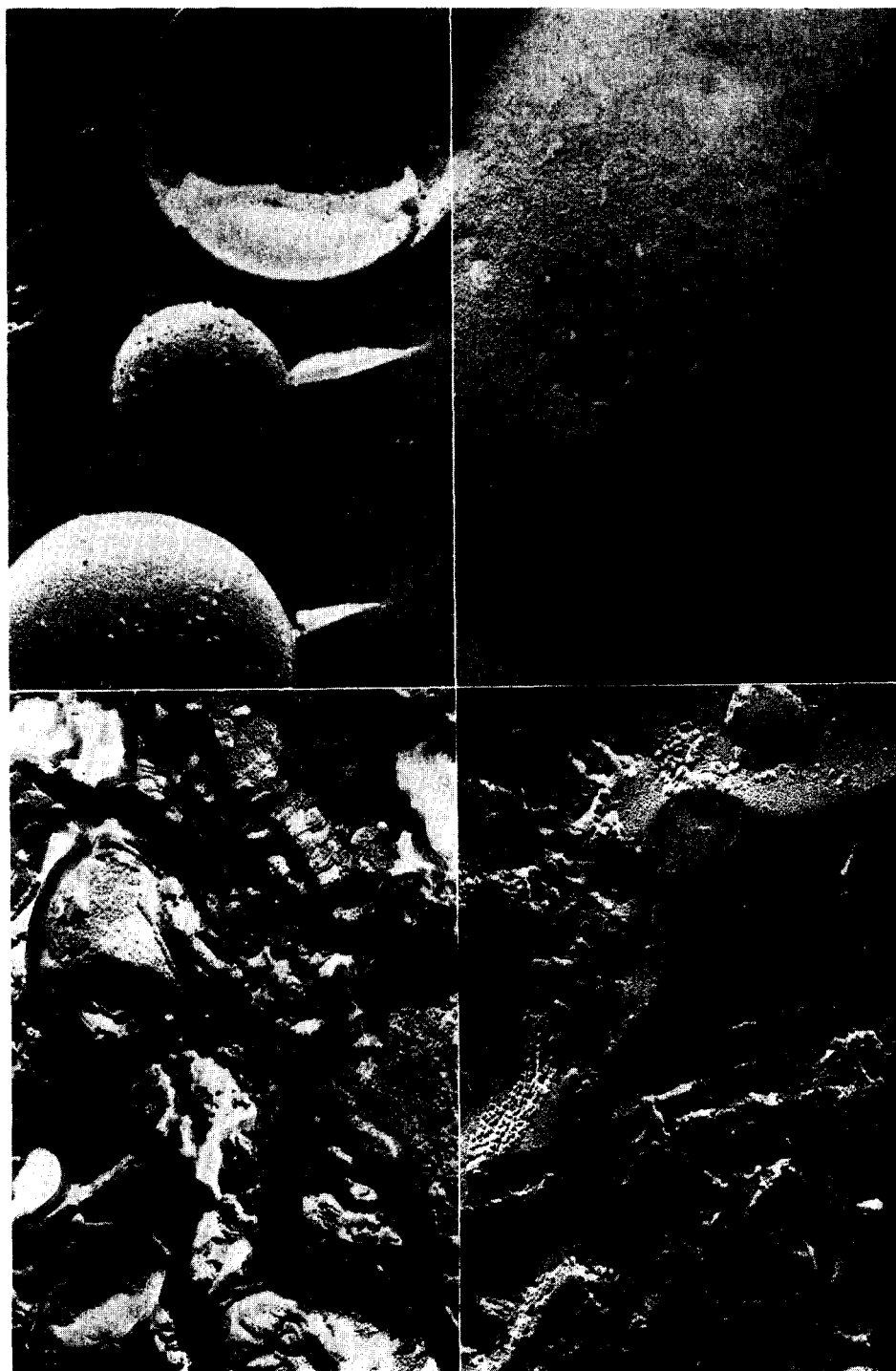


Fig. 6. Freeze-fracture electron micrographs of recombinant vesicles, prepared from a crude Triton X-100 extract and a mixture of phosphatidylethanolamine and phosphatidylserine (3 : 1) from erythrocyte membranes. The vesicles were fused by repeated freezing and thawing and incubated for 30 min at room temperature in (A) 50 mM Tris/acetic acid, pH 8.0; (B) 150 mM NaCl, 50 mM Tris/acetic acid, pH 8.0; (C) 10 mM  $\text{CaCl}_2$ , 50 mM Tris/acetic acid, pH 8.0, and (D) 50 mM Tris/acetic acid, pH 5.5. Magnification  $\times 100\ 000$ .

ghost protein (approximately 650  $\mu\text{g}$  protein/ml), consisting of band 3, 4.2, 4.5, PAS 1 and traces of band 1 and 2, and 37% of the total phospholipids (0.9  $\mu\text{mol}$   $\text{P}_i$ /ml). Vesicles showing 80- $\text{\AA}$  particles on the fracture faces were reconstituted by a method described before [12], and large fracture faces were produced by freezing-thawing of the vesicles [12]. In accordance with observations of Yu and Branton [10], recombinant vesicles made from egg phosphatidylcholine did not show changes in the lateral distribution of the particles after incubation in 150 mM NaCl, 10 mM  $\text{CaCl}_2$  or a pH 5.5 buffer (not shown). However, with a mixture of phospholipids resembling the inner monolayer of the erythrocyte membrane [5], the distribution of the particles was found to be affected by the incubation conditions. Recombinant vesicles made from a 2 : 1 : 1 mixture of phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (isolated from erythrocyte ghosts) showed particle-free areas on the fracture faces after incubation in 150 mM NaCl, 10 mM  $\text{CaCl}_2$  or at pH 5.5 buffer (Fig. 5B–D). The particle-free areas formed, had a diameter of only 750–1000  $\text{\AA}$ , and, as the particle density was much lower than in the native membrane, no aggregation was observed in the other regions of the fracture face. The incubation with 10 mM  $\text{CaCl}_2$  also caused the appearance of particles (which are larger than the normal protein particles) and pits on many of the fracture faces (Fig. 5E and F). The particles were observed only on convex fracture faces and the pits only on concave fracture faces.

Also recombinant vesicles prepared from mixtures of two phospholipids were studied. In recombinants made from a 1 : 1 mixture of phosphatidylethanolamine and phosphatidylcholine the lateral distribution of the particles was not affected by the incubation in 150 mM NaCl, 10 mM  $\text{CaCl}_2$  or a pH 5.5 buffer (not shown). In recombinant vesicles obtained from 1 : 1 mixtures of phosphatidylserine with phosphatidylcholine or phosphatidylethanolamine, the particle density was too low to detect the appearance of new particle-free areas, despite identical reconstitution conditions. Obviously the extent to which protein is incorporated into the bilayer of the vesicles is affected by the lipid composition. Recombinant vesicles made from a 3 : 1 mixture of phosphatidylethanolamine and phosphatidylserine and a concentrated Triton X-100 extract (2.2 mg protein/ml) also showed a low particle density (Fig. 6A). As shown in Fig. 6C and D both 10 mM  $\text{CaCl}_2$  and pH 5.5 caused complete disruption of these vesicles and fusion to complex lipid structures and protein aggregates. With 150 mM NaCl the vesicular structure was preserved, but on the fracture faces pits and particles were noticed (Fig. 6B).

## Discussion

Normally the particles on the erythrocyte membrane fracture faces are randomly dispersed. In some cases an altered lipid composition of the membrane was shown to be coupled with a moderate aggregation of the particles [9]. In fresh ghosts a moderate particle aggregation can be induced by calcium and basic proteins [18]. Manipulations which do not affect the particle distribution in fresh ghosts can cause aggregation after partial removal of spectrin [17]. This was explained by the precipitation of the residual spectrin under these conditions [18,19]. However, our experiments with the alkali-

treated membranes show that, after extraction of essentially all spectrin (and the other extrinsic proteins), the particles still aggregate by manipulations such as raising the ionic strength, lowering the pH or adding calcium. Therefore, precipitation of spectrin is not a satisfactory explanation for the particle aggregation induced by these conditions.

Low concentrations of the non-ionic detergent Triton X-100 cause aggregation of the particles in ghosts under conditions where spectrin is neither extracted [12,41] nor precipitated. It is possible that an interaction between spectrin and other membrane components is perturbed by the detergent, with the result that the particles might move freely; however, Bennett [21] reported that the binding of spectrin to spectrin-depleted vesicles is not abolished by the detergent. The actual cause of the particle aggregation must be related to another effect of the Triton X-100, possibly a lateral separation of lipid components as a result of preferential interactions with the detergent. Differences in the interaction between Triton X-100 and the various lipids are suggested by the differential solubilization of lipids from the membrane [42]. In a model system such differences have been demonstrated for phosphatidylcholine and sphingomyelin [43].

It is very likely that lipids are also involved in the temperature-induced particle aggregation observed in the alkali-treated membranes. In the native membrane no thermotropic transitions of phospholipids have been detected [44], presumably because of phospholipid-cholesterol interactions [45]. However, an unequal distribution of cholesterol between the two monolayers of the membrane [46] and different cholesterol affinities for various phospholipids [47,48] have been demonstrated. Of the phospholipids in the erythrocyte membrane, phosphatidylethanolamine has the lowest affinity for cholesterol (van Dijck, P.W.M., unpublished results) and it undergoes a thermotropic transition from  $+10^{\circ}\text{C}$  to  $-5^{\circ}\text{C}$  [49]. It may be speculated that removal of the extrinsic proteins affects the association of cholesterol with the components in the inner monolayer of the membrane in such a manner that allows the phosphatidylethanolamine to undergo a liquid-crystalline to gel transition. At low temperatures this would cause the exclusion of particles from regions rich in phosphatidylethanolamine\*.

Recombinant vesicles, prepared from a crude Triton X-100 extract from ghosts, show particles on the fracture faces identical to those observed in the erythrocyte membrane [10,12]. Band 3 has been identified as a constituent of these particles by reconstitutions with purified band 3 fractions [10-12]. In recombinant vesicles made from egg phosphatidylcholine, the lateral distribution of the particles was not affected by high ionic strength, low pH or the addition of calcium. This confirms the observations of Yu and Branton [10] and shows that aggregation of the particles under these conditions cannot be attributed to effects on the proteins which form the particles.

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\* This idea is consistent with the observation that quenching from  $-20^{\circ}\text{C}$  caused the appearance of large particle-free regions in recombinant vesicles prepared from a 1 : 1 mixture of phosphatidylethanolamine and phosphatidylcholine. Recombinant vesicles prepared from a mixture of 14 : 0/14 : 0-phosphatidylcholine and 18 :  $1_{\text{C}}/18 : 1_{\text{C}}$ -phosphatidylcholine showed that the particles are indeed excluded from solid lipid domains.

On the other hand, recombinant vesicles, prepared from phospholipids which are present in the inner monolayer of the erythrocyte membrane [5], showed a redistribution of the particles under conditions which induce particle aggregation in spectrin-depleted erythrocyte membranes. In recombinant vesicles made from a 2 : 1 : 1 mixture of phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine, particle-free areas were observed after incubation in 150 mM NaCl, 10 mM CaCl<sub>2</sub> or a pH 5.5 buffer. Phosphatidylserine is the component most likely to be affected by the conditions used, as the pH and the concentration of ions can strongly influence its physicochemical properties via effects on the charged polar head group [50]. Both a low pH [51] and the presence of calcium [52] have been shown to raise the phase transition temperature of phosphatidylserine dramatically. Calcium has been shown to induce lateral [53,54] and structural [51] phase separations in mixtures of phosphatidylserine and phosphatidylcholine. Also, in mixtures of phosphatidylserine and phosphatidylethanolamine calcium-induced phase separations have been observed (van Echteld, C., unpublished results). Little is known about the effects of pH and the concentration of monovalent ions on the mixing properties of phosphatidylserine and phosphatidylethanolamine. The observed rearrangement of the particles in the recombinant vesicles suggests that, in a mixture of phospholipids similar to that present in the inner monolayer of the erythrocyte membrane, conditions such as 150 mM NaCl, 10 mM CaCl<sub>2</sub> or pH 5.5 induce a lateral separation of lipid components and the formation of regions from which the particles are excluded. In the native membrane an interaction between spectrin and phosphatidylserine may interfere with this lateral separation of lipids which is induced by environmental conditions and may prevent the aggregation of the particles. A clear interaction between spectrin and phosphatidylserine has been shown recently [24], whereas less interaction was detected with phosphatidylcholine [23] and phosphatidylethanolamine (Mombers, C., unpublished results). Phase separations in mixtures of phosphatidylserine and phosphatidylcholine were found to be reduced by the presence of spectrin [24]. Removal of spectrin from the erythrocyte membrane may expose the phosphatidylserine molecules and thereby lead to particle aggregation through effects on this lipid component. A precipitation of residual spectrin [18] could enhance this effect by clustering the phosphatidylserines still interacting with this spectrin. In the absence of spectrin the lipid components in the inner monolayer of the erythrocyte membrane may be expected to respond to the various manipulations in a way comparable to the lipids in the recombinant vesicles. The same conditions were indeed shown to cause a redistribution of the particles in the alkali-treated membranes and in the recombinant vesicles prepared from the inner monolayer phospholipids. That morphologically the effects observed, are somewhat different may be due to the more complex organization of the lipids in the erythrocyte membrane. From observations on recombinant vesicles Yu and Branton [10] concluded that spectrin-actin was bound to integral proteins, and that this association caused the particles to aggregate at low pH. However, the recombinant vesicles were prepared from a crude Triton X-100 extract which contains a considerable amount of phospholipids, including phosphatidylserine [32,42]. This could also be responsible for the binding of spectrin-actin. Exactly how the bound

spectrin-actin induces aggregation of the particles in this system at low pH is not clear.

In the recombinant vesicles prepared from the inner monolayer phospholipids of the erythrocyte membrane, large particles and corresponding pits were observed upon addition of calcium. The phosphatidylethanolamine of the erythrocyte membrane exhibits a bilayer to hexagonal ( $H_{11}$ ) transition at 10°C [55]. Therefore at 23°C it will tend to adopt a non-bilayer configuration when it is laterally separated from the other lipid components. This may cause the formation of invaginations in the bilayer [56], which show up in the fracture plane as particles with corresponding pits. Also the effects of high ionic strength, low pH and calcium on the 3 : 1 phosphatidylethanolamine/phosphatidylserine recombinant vesicles can be accounted for by the tendency of the phosphatidylethanolamine to adopt a non-bilayer configuration. These observations strengthen the idea that, under certain conditions, alternative lipid structures may occur in the native membrane too. As the lateral distribution of particles in the native membrane is controlled by spectrin [17], the formation of such non-bilayer configurations may also be controlled by the interaction of spectrin with the inner monolayer phospholipids, especially with the phosphatidylserine. Perturbation of the interaction of spectrin with the inner monolayer could enhance the formation of transitory non-bilayer configurations, and consequently enhance processes such as flip-flop, membrane fusion and blebbing off. Indeed, in chicken erythrocytes dephosphorylation of membrane proteins, including spectrin, has been correlated with aggregation of the intramembrane particles and with increased exposure of phosphatidylethanolamine at the outer face of the membrane [57]. In human erythrocytes modification of sulfhydryl groups of spectrin has been associated with a movement of phosphatidylethanolamine and phosphatidylserine to the outer layer of the membrane [25]. Both ATP depletion and increased levels of calcium at the inner monolayer have been shown to cause increased fusion [58,59] and the release of vesicles [60–63]. Lateral separation of lipid components in the inner monolayer of the erythrocyte membrane can account for the aggregation of the intramembrane particles by a variety of conditions. Most likely in the native membrane aggregation of the particles by mono- and divalent cations and low pH is prevented by the interaction of phosphatidylserine with spectrin. Moreover this interaction is thought to control the occurrence of transitory non-bilayer configurations of phosphatidylethanolamine.

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